

# Formulation and evaluation of a herbal antibacterial cream from ethyl acetate extract of leaves of *Spinacia oleracea* Linn. against *Aeromonas* skin and soft tissue infections

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## Abstract

**Introduction:** Skin and soft tissue infections still remain a major challenge in assessing the etiological and severity conditions. The rapid onset of cellulitis in the setting of soft tissue trauma and exposure to water results in the possibility of infection with this organism. In our previous study, we carried out the antibacterial activity of successive extracts of leaves of *Spinacia oleracea* against various Gram-positive and Gram-negative bacteria. Of that, the successive ethyl acetate extract showed a significant antibacterial effect over *Aeromonas hydrophila*. Hence, in the present work, the active ethyl acetate extract was formulated into a suitable herbal cream and evaluated for its physical, chemical, and stability parameters. **Materials and Methods:** The shade dried, powdered plant material was subjected to successive Soxhlet extraction with n-hexane followed by ethyl acetate. Preliminary phytochemical screening along with thin layer and high-performance thin-layer chromatography of the active ethyl acetate extract was established. Four different formulations F1, F2, F3, and F4 were subjected to various evaluations including physical and chemical parameters and stability testing. **Results:** Formulation F2 and F3 found to be more stable, while remaining formulations were not stable and resulted in breakdown of the emulsion when stored for long time. These formulations F2 and F3 had almost constant pH and were homogeneous, emollient, and non-greasy and can be easily removed after the application. The stable formulations were safe in respect to skin irritation and allergic sensitization. **Conclusion:** Thus, the study validates the formulation and stability studies carried out on the active ethyl acetate extract against *Aeromonas* skin and soft tissue infections.

**Key words:** Antibacterial, cream, formulation, spinach, successive extracts

## INTRODUCTION

*Aeromonas hydrophila* of family Vibrionaceae is the most commonly isolated species associated with human infections including gastrointestinal tract illness, soft tissue infections, pneumonia, endocarditis, meningitis, osteomyelitis, and septic arthritis. The treatment for soft tissue infections caused by this organism includes both medical and surgical regimens. Incision and drainage are required for puncture wounds, and after surgical decompression, parenteral and oral antibiotics is an essential part of the treatment. While clinical isolates of *Aeromonas* are susceptible to a wide range of antibiotics, they are universally resistant to penicillin, ampicillin, carbenicillin, and cefazolin.<sup>[1]</sup>

Although *Spinacia oleracea* Linn., (Chenopodiaceae), a leafy green vegetable most often used as food, it possesses medicinal value as well. Most often used as a food, it has medicinal value as well. Spinach is packed with vitamins such as Vitamin C, Vitamin A, and Vitamin E and minerals such as magnesium, manganese, iron, calcium, and folic

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acid. Spinach is also a good source of chlorophyll, which is known to aid in digestion. Spinach is also rich in the carotenoids beta-carotene and lutein.<sup>[2-5]</sup> It is a good source of the bioflavonoid quercetin with many other flavonoids which exhibits antioxidant, antiproliferative, anti-inflammatory, antihistaminic, CNS depressant, protection against gamma radiation, and hepatoprotective properties.<sup>[6-12]</sup> Our previous study revealed that ethyl acetate extract showed a significant antibacterial activity over the other successive extracts against *A. hydrophila*.<sup>[13]</sup> Hence, the active ethyl acetate extract was formulated into a herbal cream and evaluated for its physical, chemical, and stability parameters.

## MATERIALS AND METHODS

### Plant Material

Fresh leaves of *S. oleracea* Linn. were collected from Uthiramerur, Chengalpattu District, Tamil Nadu, during October. Fully grown leaves were collected during a fine dry weather in a shed for 3 weeks. The plant was identified and authenticated by Prof. Dr. Jayaraman, Plant Anatomy Research Centre, Tambaram. The shade dried leaves were coarsely powdered and used for further studies. A voucher specimen has been reserved in the Department of Pharmacognosy, SRM College of Pharmacy, SRM University, Kattankulathur.

### Preparation of Extracts<sup>[14]</sup>

Extraction was performed by successive Soxhlet extraction of coarsely powdered leaves of *S. oleracea* Linn., with n-hexane followed by ethyl acetate. The ethyl acetate extract was concentrated by distilling off the solvent and evaporated to dryness and the percentage yield was calculated.

### Phytochemical Analysis<sup>[15-20]</sup>

Preliminary phytochemical screening was carried out to identify the presence of various phytochemical constituents. Furthermore, thin-layer chromatography and high-performance thin-layer chromatography of ethyl acetate extract were established.

### Formulation and Evaluation Studies

#### Formulation of cream<sup>[21]</sup>

An oil-in-water (O/W) emulsion-based cream of *S. oleracea* Linn. was formulated. The emulsifier (stearic acid) and other oil-soluble components (cetyl alcohol and spermaceti) were dissolved in the oil phase (Part A) and heated to 75°C. The preservatives and other water-soluble components, namely triethanolamine, benzyl alcohol, glycerine, ethyl acetate extract of *S. oleracea* Linn., were dissolved in the aqueous phase (Part B)

and heated to 75°C. After heating, the aqueous phase was added in portions to the oil phase with continuous stirring until cooling of emulsifier takes place. The formula for the cream is given in Table 1. The base formula contained excess fat which produced a greasy sense on usage, turbidity, and its low consistency. At first, the proportions of the oily phase components were changed and four formulations were made. Finally, the best formulation was chosen according to the results of different chemical and physical tests for further stability testing.

### Evaluation of Cream

The cream was evaluated for the following parameters.

#### Determination of physical parameters

The appearance of the cream was judged by its color, pearlscence and roughness, and graded.<sup>[22]</sup> The pH meter was calibrated using standard buffer solution. About 0.5 g of the cream was weighed and dissolved in 50.0 mL of distilled water and its pH was measured. Viscosity of the formulation was determined by Brookfield viscometer at 100 rpm, using spindle no 7. The formulations were tested for the homogeneity by visual appearance and touch.

#### Determination of robustness<sup>[22]</sup>

It includes following determination of spreadability.

About 3 g of the sample was applied in between two glass slides and they were pressed together to obtain a film of uniform thickness by placing 1000 g weight for 5 min after which 10 g weight was added to the pan, and the top plate was subjected to pull with the help of string attached to the hook. The time in which the upper glass slide moves over the lower plate to cover a distance of 10 cm is noted. The spreadability (S) can be calculated using the formula.

$$S = m \times L/t$$

Where,

**Table 1:** The composition and the amount of ingredients used to make 10 g of EAESO herbal cream

Components	Ingredients	F1	F2	F3	F4
Oil phase	Stearic acid	1.5	1	1	0.5
	Cetyl alcohol	1.5	1	0.5	0.5
	Almond oil	0.5	0.5	0.5	0.5
Aqueous phase	Active extract	0.1	0.1	0.1	0.1
	Triethanolamine	0.2	0.2	0.2	0.2
	Glycerin	0.5	0.5	0.5	0.5
	Benzyl alcohol	0.2	0.2	0.2	0.2
	Water	qs	qs	qs	qs

S - spreadability.  
m - weight tied to upper glass slide.  
l - length moved on a glass slide.  
t - time taken.

The determinations were carried out in triplicate and the average of three readings was recorded.

#### **Determination of wetness, type of smear, and emolliency**

It was determined by applying cream on skin surface of human volunteer. After application of cream, the type of film or smear formed on the skin was checked.<sup>[22]</sup> Emolliency, slipperiness, and amount of residue left after the application of fixed amounts of cream was checked. The ease of removal of the cream applied was examined by washing the applied part with tap water.

#### **Determination of type of emulsion<sup>[22]</sup>**

##### ***Dilution test***

Dilution test determines the type of emulsion formed in the formulation. The formulation is diluted either with oil or water. If the emulsion is O/W type and it is diluted with water, it will remain stable as water is the dispersion medium. When the oil in water type cream is diluted with water, it remains stable. If it is diluted with oil, the emulsion breaks. The water in oil type cream holds good when diluted with oily liquid and breaks upon addition of water. O/W emulsion can easily be diluted with an aqueous solvent, whereas water in oil emulsion can be diluted with an oily liquid.

##### ***Dye solubility test***

In this test, an emulsion is mixed with a water-soluble dye (amaranth) and observed under the microscope. If the continuous phase appears red, it means that the emulsion is O/W type as the water is in the external phase and the dye will dissolve in it to give color. If the scattered globules appear red and continuous phase colorless, then it is w/o type. Similarly, if an oil-soluble dye (Scarlet red C or Sudan III) is added to an emulsion and the continuous phase appears red, then it is w/o emulsion.

#### **Determination of chemical parameters<sup>[23]</sup>**

The acid value and saponification value of the prepared formulations were found.

##### ***Irritancy test<sup>[24]</sup>***

Mark an area (1 sq.cm) on the left hand dorsal surface. The cream was applied to the specified area and time was noted. Irritancy, erythema, and edema were checked if any for regular intervals up to 24 h and reported.

#### **Stability tests<sup>[25,26]</sup>**

##### ***Agitation test***

5 g of the non-aqueous cream was filled in a container and container was placed on a reciprocating shaker. The container was shaken at room temperature approximately 60 cycles per

minute at for 24 h. After 24 h, container was removed and cream was observed for any signs of phase separation.<sup>[11]</sup>

##### ***Centrifugation test***

Centrifugation test was performed immediately after formulation of the non-aqueous cream. 5 g of non-aqueous cream was filled in a centrifuge tube. The tubes containing non-aqueous cream were subjected to centrifugation at 3500 rpm for 30 min. Cream was observed for any signs of phase separation after the test.

##### ***Accelerated stability testing<sup>[27,28]</sup>***

Accelerated stability testing of prepared formulations was conducted for two most stable formulations at room temperature and at 40°C ± 1°C for 7 days. They were formulation number 2 and 3 at 40°C ± 1°C for 20 days. The formulations were kept both at room and elevated temperature and observed on 0<sup>th</sup>, 5<sup>th</sup>, 10<sup>th</sup>, 15<sup>th</sup>, and 20<sup>th</sup> days.

## **RESULTS**

### **Phytochemical Analysis**

Successive extractive values revealed the solubility and polarity particulars of the metabolites in the leaf powder. Percentage yield of n-hexane and ethyl acetate extract was found to be 1.86% w/w and 4.22% w/w respectively. Qualitative preliminary phytochemical analysis was performed initially with different respective chemical detecting agent to detect the phytoconstituent nature and their presence in each extract and powder. n-hexane extract showed the presence of lipids. Ethyl acetate extract was found to contain proteins, steroids, alkaloids, flavonoids, phenols, and tannins.

Qualitative chromatographic analysis of these extracts using thin-layer chromatography was performed to separate and identify the single or mixture of constituents in each extract. Three spots were found active in ethyl acetate extract (0.54, 0.59, and 0.79) under daylight in chloroform: methanol (9:1). High-performance thin-layer chromatography was scanned at 400 nm with the best solvent to detect the maximum number of components and peak abundance qualitatively and quantitatively at higher resolution. The fingerprint of ethyl acetate extract was given in Figure 1.

### **Evaluation of Cream**

#### **Determination of physical parameters**

No change in color of cream was observed even after storing for a considerable period. All the formulations of cream were shown pH nearer to skin pH in the range of 5.6–6.2. The viscosity of cream was in the range of 28005–28985 cps which indicates that the cream is easily spreadable by small amounts of shear. However, F2 and F3 show good spreadable property than other formulations. All

formulations produce uniform distribution of extracts in cream. This was confirmed by visual appearance and touch. Emolliency, slipperiness, and amount of residue left after the application of fixed amount of cream was found [Table 2]. The dye test confirms that all formulations were O/W type emulsion cream. However, formulation (F2) shows more stability in O/W type emulsion.

### Acid value and saponification value

The results of acid and saponification value of all formulations of cream are presented in Table 3 and showed satisfactorily values.

### Irritancy test

The formulations showed no redness, edema, inflammation, and irritation during irritancy studies. Hence the formulations are safer to skin [Table 4].

### Stability test

The phase separation and viscosity parameters after agitation and centrifugation were tabulated in Table 5.

### Accelerated stability testing

The changes in the physical parameters of F2 and F3 were observed on 0<sup>th</sup>, 5<sup>th</sup>, 10<sup>th</sup>, 15<sup>th</sup>, and 20<sup>th</sup> days were tabulated in Table 6.

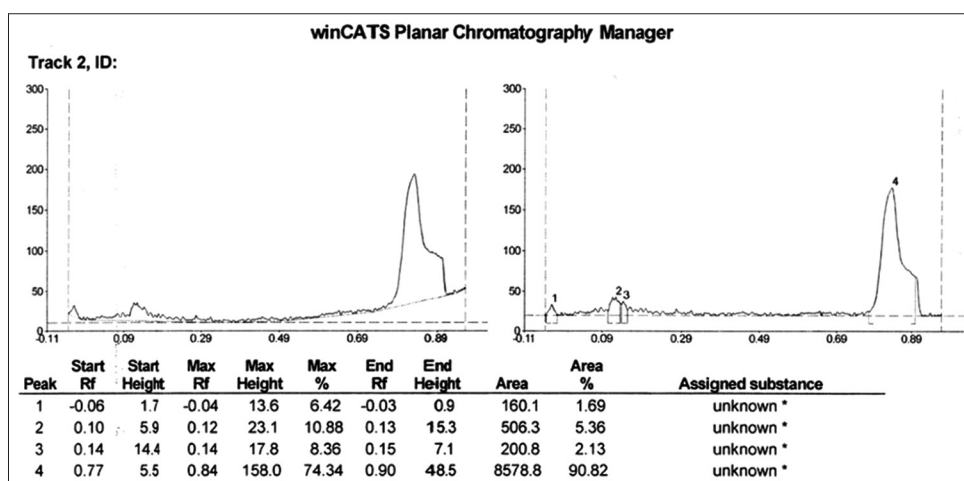


Figure 1: High-performance thin-layer chromatography profile on the ethyl acetate extract of *Spinacia oleracea* Linn.

Table 2: Evaluation of physical parameters of various formulations of EAESO herbal cream

Parameters	F1	F2	F3	F4
Appearance	Greenish	Greenish	Greenish	Greenish
pH	5.8	6.2	6.2	6.4
Viscosity	28059	28489	28932	28845
Homogeneity				
By visual	Homogenous	Homogenous	Homogenous	Homogenous
By touch	Smooth and consistent	Smooth and consistent	Smooth and consistent	Smooth and consistent
Robustness				
Spreadability	Easily spreadable	Easily spreadable	Easily spreadable	Easily spreadable
Wetness	Moisturises skin surface	Moisturises skin surface	Moisturises skin surface	Moisturises skin surface
Type of smear	Slightly greasy	Non-greasy	Non-greasy	Non-greasy
Emolliency	No residue left	No residue left	No residue left	No residue left
Dilution test	O/W type of emulsion	O/W type of emulsion	O/W type of emulsion	O/W type of emulsion
Dye solubility test	O/W type of emulsion	O/W type of emulsion	O/W type of emulsion	O/W type of emulsion

Table 3: Determination of acid value and saponification value of formulations of EAESO cream

Parameter	F1	F2	F3	F4
Acid value	6.8	6.4	6.4	5.8
Saponification value	30.1	23.2	27.4	26.2

Table 4: Types of adverse effect of formulations

Parameter	F1	F2	F3	F4
Irritant	Nil	Nil	Nil	Nil
Erythema	Nil	Nil	Nil	Nil
Edema	Nil	Nil	Nil	Nil

**Table 5:** Stability study of formulations after agitation and centrifugation

Stability tests	Parameters	Evaluation parameters of trial batches				
		F1	F2	F3	F4	Remarks
Agitation	Phase separation	No	No	No	No	No phase separation
	Viscosity (Cps)	28008	28456	28968	28869	Slight change in viscosity
Centrifugation	Phase separation	No	No	No	No	No phase separation
	Viscosity (Cps)	28569	28496	28532	28759	Slight change in viscosity

**Table 6:** Physical parameters of F2 and F3 cream on room and accelerated temperature

Day	Formulation	Temperature	pH	Homogeneity	Appearance	Spreadability	After feel	Type of smear	Removal
0 <sup>th</sup>	F2	RT	6.2	Good	No color change	Good	Emollient	Non-greasy	Easy
		40°C±1°C	6.3	Good	No color change	Good	Emollient	Non-greasy	Easy
	F3	RT	6.2	Good	No color change	Good	Emollient	Non-greasy	Easy
		40°C±1°C	6.2	Good	No color change	Good	Emollient	Non-greasy	Easy
5 <sup>th</sup>	F2	RT	6.3	Good	No color change	Good	Emollient	Non-greasy	Easy
		40°C±1°C	6.3	Good	No color change	Good	Emollient	Non-greasy	Easy
	F3	RT	6.2	Good	No color change	Good	Emollient	Non-greasy	Easy
		40°C±1°C	6.3	Good	No color change	Good	Emollient	Non-greasy	Easy
10 <sup>th</sup>	F2	RT	6.3	Good	No color change	Good	Emollient	Non-greasy	Easy
		40°C±1°C	6.3	Satisfactory	No color change	Good	Emollient	Non-greasy	Easy
	F3	RT	6.3	Good	No color change	Good	Emollient	Non-greasy	Easy
		40°C±1°C	6.5	Satisfactory	No color change	Good	Emollient	Non-greasy	Easy
15 <sup>th</sup>	F2	RT	6.4	Satisfactory	No color change	Good	Emollient	Non-greasy	Easy
		40°C±1°C	6.4	Satisfactory	No color change	Good	Emollient	Non-greasy	Easy
	F3	RT	6.3	Good	No color change	Good	Emollient	Non-greasy	Easy
		40°C±1°C	6.6	Good	No color change	Good	Emollient	Non-greasy	Easy
20 <sup>th</sup>	F2	RT	6.5	Good	No color change	Good	Emollient	Non-greasy	Easy
		40°C±1°C	6.5	Good	No color change	Good	Emollient	Non-greasy	Easy
	F3	RT	6.3	Good	No color change	Good	Emollient	Non-greasy	Easy
		40°C±1°C	6.6	Good	No color change	Good	Emollient	Non-greasy	Easy

## DISCUSSION

Plant extracts with antibacterial activity have been formulated as topical creams. It has been previously reported that formulation of *Zataria multiflora* extract as topical cream may lead to enhancement of stability and acceptability of the active ingredient, while the antimicrobial activity remains considerable.<sup>[29]</sup> It has been shown that aqueous extract of *Ziziphus spina* leaves had antibacterial activity and was formulated as a topical formulation to treat skin infections.<sup>[30]</sup> Handali *et al.* formulated and evaluated an antibacterial cream from *Oxalis corniculata*.<sup>[31]</sup> Proper standardization techniques and quality control of the herbal preparations have to be carried out consistently.<sup>[32]</sup> *S. oleracea* Linn. has already been proven for its antibacterial study. The active ethyl acetate extract of leaves of *S. oleracea* Linn. extract was formulated into a suitable herbal cream. The base formula due to its excess fat content resulted in a greasy sense on usage, turbidity, and poor

consistency. To overcome these problems, the proportion of the oil phase components was changed. Our results indicated that the formulation F2 and F3 was found to be more stable, while remaining formulations resulted in breakdown of the emulsion when stored for a longer period. These formulations F2 and F3 had almost constant pH and were homogeneous, emollient, and non-greasy and can be easily removed after the application and were safe in respect to skin irritation and allergic sensitization. Moving to a higher note, evaluation of cutaneous permeation and *in vivo* efficacy of the formulations are necessary to confirm their usage for the treatment of various *Aeromonas* skin and soft tissue infections.

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